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Modulation of hair cell efferents

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Abstract

Outer hair cells (OHCs) amplify the sound-evoked motion of the basilar membrane to enhance acoustic sensitivity and frequency selectivity. Medial olivocochlear (MOC) efferents inhibit OHCs to reduce the sound-evoked response of cochlear afferent neurons. OHC inhibition occurs through the activation of postsynaptic $\alpha 9\alpha 10$ nicotinic receptors tightly coupled to calcium-dependent SK2 channels that hyperpolarize the hair cell. MOC neurons are cholinergic but a number of other neurotransmitters and neuromodulators have been proposed to participate in efferent transmission, with emerging evidence for both pre- and postsynaptic effects. Cochlear inhibition *in vivo* is maximized by repetitive activation of the efferents, reflecting facilitation and summation of transmitter release onto outer hair cells. This review summarizes recent studies on cellular and molecular mechanisms of cholinergic inhibition and the regulation of those molecular components, in particular the involvement of intracellular calcium. Facilitation at the efferent synapse is compared in a variety of animals, as well as other possible mechanisms of modulation of ACh release. These results suggest that short-term plasticity contributes to effective cholinergic inhibition of hair cells.

1. Introduction

Mechanosensory hair cells in the organ of Corti send acoustic information to the brain through synapses with peripheral afferent neurons. In return, feedback provided by efferent neurons located in the brainstem and projecting to the cochlea modulates that afferent activity. Efferent inhibition of the auditory end-organs was first shown by Galambos (1956). Electrical stimulation of the olivocochlear axons reduced the amplitude of the compound action potential (CAP) recorded extracellularly from the VIIIth nerve in response to an acoustic click or brief tone burst. It is now widely accepted that this CAP reduction resulted from medial olivocochlear (MOC) efferent inhibition of the active electromotile response of OHCs that amplifies basilar membrane motion to increase acoustic sensitivity (Brownell et al., 1985; Ashmore, 1987) for review see (Guinan and Stankovic, 1996; Cooper and Guinan, 2006; Ashmore, 2008).

In mammals, the efferent neurons can be classified into two anatomically and functionally distinct groups named according to their origin in the lateral or medial region of the superior olivary complex (Warr and Guinan, 1979): lateral olivocochlear (LOC) efferents project to the region near the inner hair cells (IHCs) and terminate on the dendrites of type I auditory afferents postsynaptic to the IHCs. Medial olivocochlear (MOC) efferents originate in the

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medial and rostral region of the superior olivary complex and send thicker myelinated axons to innervate predominantly outer hair cells (OHCs) in the mature cochlea as well the IHCs of pre-hearing animals (see for review (Simmons, 2002; Bruce et al., 2000).

This review will summarize physiological studies of efferent inhibition in hair cells of vertebrates ranging from fish to mammals. Studies in the mammalian cochlea focused initially on the transient MOC synapses on immature inner hair cells (IHCs), but improving methodologies have increased attention to older OHCs. However, postsynaptic recordings of LOC effects in type I afferents are only beginning, and so are not available for this review. The interested Reader is referred to previous studies of LOC function (Ruel et al., 2007). The mechanism of cholinergic inhibition is well-conserved among vertebrate hair cells, mediated by an unusual ionotropic ACh receptor and associated calcium-activated potassium channels. In addition, efferent terminals may contain other neurotransmitters, receptors and channels that could modulate synaptic strength. These candidate signaling molecules will be summarized first. Secondly, we will discuss emerging knowledge on the mechanics of synaptic transmission and its modulation by neurotransmitters or small proteins. Short-term facilitation of inhibition has been observed in several species, and may be a fundamental property of efferent feedback. Finally, we review *in vivo* olivocochlear physiology, in particular those studies providing information on the discharge rate of MOC neurons to assess further the role of activity-dependent plasticity in olivocochlear function.

2. Cellular mechanism of MOC efferent inhibition

Although recent studies have concentrated on efferent inhibition of mammalian cochlear hair cells, the initial descriptions of cellular mechanisms came from studies in non-mammalian vertebrates. In the lateral line organ of fish (the burbot) electrical stimulation of efferent fibers evoked inhibitory post-synaptic potentials (IPSPs) that hyperpolarized the hair cells and reduced the amplitude of spontaneous or evoked EPSPs recorded from afferent nerve terminals (Flock and Russell, 1976). Similar results were observed in frog saccular hair cells (Ashmore and Russell, 1983) as well as in the turtle basilar papilla where hair cell hyperpolarization and afferent inhibition were strengthened by higher frequency efferent stimulation (Art et al., 1984). Furthermore, efferent inhibition decreased the receptor potential specifically at the characteristic frequency, effectively 'detuning' the hair cell. Although this effect is related to the electrical tuning mechanism in turtle hair cells (Art et al., 1985), efferent inhibition in the mammalian cochlea also reduces the sharpness of tuning, by suppressing the electromotility of OHCs (Brown et al., 1983; Brown and Nuttall, 1984). Thus despite substantial differences in cellular organization and tuning mechanisms, the outcome is similar among vertebrates; efferent inhibition desensitizes and detunes the auditory end-organ. At the cellular level, the ionic mechanism of cholinergic inhibition and the morphology of the MOC efferent synapse are strongly conserved among vertebrates. Less certain however is what other transmitters and receptors are present at efferent synapses, and how synaptic strength can be modulated.

2.1. Neurotransmitter and postsynaptic molecular components

The main transmitter of MOC efferents is acetylcholine (ACh) (Eybalin, 1993). First nominated on the basis of cochlear cholinesterase histochemistry (Churchill et al., 1956) pharmacological evidence was obtained in the lateral line of *Xenopus laevis* (Russell, 1971) followed by studies in the guinea pig cochlea (Norris and Guth, 1974; Fex and Wenthold, 1976). It is now established that efferent release of ACh activates $\alpha 9\alpha 10$ -containing ionotropic ACh receptors (nAChRs) (Elgoyhen et al., 1994, 2001) with the subsequent opening of small conductance calcium-activated SK2 channels (Dulon et al., 1998; Oliver et al., 2000; Nie et al., 2004). 'Knockout' mice missing either $\alpha 9$ or $\alpha 10$ fail to show suppression of cochlear responses (compound action potentials (CAP), distortion product

otoacoustic emissions (DPOAEs)) during efferent fiber stimulation (Vetter et al., 1999, 2007) and their hair cells no longer respond to local application of ACh (Vetter et al., 2007) (Fig. 1). Metabotropic ACh receptors also have been localized to different cochlear structures but their expression by hair cells remains uncertain (Heilbronn et al., 1995; Safieddine et al., 1996; Khan et al., 2002; for review see (Puel, 1995)). Rather, RT-PCR and immunolabeling suggest that metabotropic AChRs may be expressed on the MOC terminals (Bartolami et al., 1993; Safieddine et al., 1996; Maison et al., 2010).

MOC terminals also immunolabel for GABA, and several peptides (CGRP, opioids) (Fex and Altschuler, 1981; Altschuler et al., 1984; Fex and Altschuler, 1984; Whitlon and Sobkowicz, 1989; Eybalin, 1993; Vetter et al., 1991). Double immunolabeling of mouse cochlea for the vesicular acetylcholine transporter (VAT), glutamic acid decarboxylase (GAD, for synthesis of GABA) and CGRP demonstrated the co-localization of GABA and CGRP with ACh in all MOC terminals on OHCs, independent of their apico-basal location, suggesting a universal role for these neuro-active compounds (Maison et al., 2003a,b). However, to date the only demonstrated efferent synaptic signals are cholinergic (see following section), thus GABA and CGRP might serve as co-transmitters to modulate the strength and efficacy of MOC transmission.

2.2. Hair cell responses to efferent neurotransmitters

Efferent activation caused hyperpolarizing IPSPs in hair cells of non-mammalian vertebrates. In the turtle these IPSPs were biphasic, with a small depolarization preceding the larger and longer-lasting hyperpolarization (Art et al., 1984). Chicken auditory hair cells exhibit a similarly-shaped biphasic change in membrane conductance in response to local application of ACh (Fuchs and Murrow, 1992a,b). Biphasic synaptic currents have been described in neonatal rat IHCs (Glowatzki and Fuchs, 2000; Katz et al., 2004; Gómez-Casati et al., 2005; Goutman et al., 2005) as well as in older OHCs (Nenov et al., 1996; Oliver et al., 2000; Lioudyno et al., 2004). The kinetics and voltage-dependence of the synaptic currents, as well as experiments with internal calcium buffers, support a ‘two-channel’ hypothesis whereby calcium influx through the AChR triggers potassium efflux through associated SK2 channels (Fuchs and Murrow, 1992a,b; Blanchet et al., 1996; Evans, 1996; Yuhas and Fuchs, 1999; Oliver et al., 2000). Consistent with this hypothesis, the mammalian hair cell AChR has a relatively high permeability to calcium (Weisstaub et al., 2002; Gómez-Casati et al., 2005; Plazas et al., 2005).

Regarding the possibility of GABA as an efferent transmitter, *ex vivo* experiments have shown a potassium induced release of GABA in the guinea pig cochlea (Bobbin et al., 1990a,b), while local application of GABA hyperpolarized apical OHCs from the same species (Gitter and Zenner, 1992; Plinkert et al., 1993) and reduced OHC electromotility and stiffness (Sziklai et al., 1996; Batta et al., 2004). However, locally-applied GABA had no effect on OHCs in other studies (Bobbin et al., 1990a,b; Evans et al., 1996). OHCs may express postsynaptic GABA_A receptors, although the overall pattern of expression and the role of GABAergic transmission in efferent feedback remains unclear (Plinkert et al., 1989, 1993; Drescher et al., 1993; Maison et al., 2006). Likewise, the role of CGRP, most likely as an efferent neuromodulator, remains unresolved. MOC function (distortion product suppression) and resistance to acoustic injury were unchanged in an α CGRP knockout mouse (Maison et al., 2003a,b). Finally, dynorphin and endomorphin opioid peptides have been shown to reduce the synaptic currents due to ACh released from the efferent terminals by acting directly on α 9 α 10-containing nicotinic receptors in immature IHCs (Lioudyno et al., 2002). However, while there is a general consensus regarding the localization of opioid peptides in LOC efferents (Safieddine and Eybalin, 1992; Safieddine et al., 1997), their presence in MOC efferent terminals remains controversial (Altschuler et al., 1984, 1985; Hoffman et al., 1984; Hoffman et al., 1985).

2.3. Postsynaptic ultrastructure and calcium signaling

A consistent feature of the efferent synapse is the near-membrane cistern that lays less than 20 nm from the plasma membrane within the hair cell and is co-extensive with the efferent terminal (Saito, 1980).

The synaptic cistern (SC) appears as an 'extra' pair of membranes in cross-section (Engstrom and Wersall, 1958) that form an enclosed flattened sac (without ribosomes) in serial sections (Smith and Sjostrand, 1961). Similar structures are present in neurons from cerebral cortex and spinal cord as well as in numerous other regions of the brain (Rosenbluth, 1962; Yamamoto et al., 1991). On the basis of their similarity to the sarcoplasmic reticulum (SR)/T-tubule complex in striated muscle (Parekh and Penner, 1997; Berridge, 1998; Patterson et al., 1999), SCs have been hypothesized to be a source of calcium mobilization through calcium-induced calcium release (CICR). Such a mechanism could contribute to the seconds-long time course of K^+ currents in early descriptions of the effect of ACh on cochlear hair cells (Housley et al., 1990; Shigemoto and Ohmori, 1991; Kakehata et al., 1993). ACh-induced CICR is believed to be mediated by the calcium-activated ryanodine receptor 1 (RyR1) expressed by OHCs in the rat (Lioudyno et al., 2004) and localized to the efferent synapse (Grant et al., 2006).

Various pharmacological studies support the idea of a more complex postsynaptic mechanism than the 'two channel' hypothesis. Store-active compounds commonly used to probe CICR including ryanodine (an agonist of the RyR), thapsigargin and cyclopiazide acid (membrane-permeable inhibitors of SERCA pumps) and caffeine (a modulator of RyR opening) lead to the enhancement of efferent inhibition *in vivo* (Sridhar et al., 1997; Bobbin, 2002). These compounds also modulate the calcium-activated K^+ current recorded in OHCs when ACh acts over longer time scales (exogenous application for 1–10 s) (Evans et al., 2000; Lioudyno et al., 2004).

In addition to the storage and release of calcium, the synaptic cistern may shape the kinetics of hair cell inhibition by other means. For example, the SC could constitute a diffusion barrier to isolate the efferent molecular components from cytoplasmic calcium buffers (Hackney et al., 2005) or be itself a fixed calcium binding system. For example, the build-up and gradual dispersal of calcium in the restricted diffusion space between cisternal and plasma membranes could contribute to the long lasting hyperpolarization observed after strong efferent inhibition (Goutman et al., 2005). Additionally, the close association often seen between the synaptic cistern and mitochondria also suggests a function in the efferent synapse for this organelle (Rosenbluth, 1962) (Fig. 2). Finally, the early appearance of cisterns (postnatal day 4) in rat OHCs (Bruce et al., 2000) before efferent function is fully established suggests that cisterns also may serve a role in synaptic maturation.

2.4. Electrical stimulation of olivocochlear efferents induces slow and fast effects in vivo

Electrical stimulation of the MOC efferents suppresses the sound-evoked CAP within 100 ms (Galambos, 1956; Wiederhold and Kiang, 1970). This fast effect is mediated by the cholinergic-induced hyperpolarization of OHCs and the subsequent decrease in amplification of basilar membrane motion (Murugasu and Russell, 1996; Russell and Murugasu, 1997).

However during continuous efferent stimulation, there is an additional slow reduction of sound-evoked afferent discharges and basilar membrane motion (Cooper and Guinan, 2003); (see for review (Cooper and Guinan, 2006)) that builds up and decays over tens of seconds. Both fast and slow inhibition depend on an intact olivocochlear bundle, result from activation of the hair cell's nAChR, and reach their maximum for efferent stimulation rates of 200–400/sec (Sridhar et al., 1995).

Fast and slow efferent effects on basilar motion are likely to reflect different consequences of OHC inhibition. Fast inhibition may correspond to the immediate effect of the synaptic hyperpolarization and conductance increase on OHC electromotility (Rabbitt et al., 2009). The slow form of inhibition is believed to result from changes in the axial stiffness of OHCs (Dallos et al., 1997; He et al., 2003) perhaps mediated by a wave of calcium-induced calcium release from the synaptic and lateral cisterns (Sridhar et al., 1997).

3. Modulation of efferent inhibition: short-term plasticity

3.1. Short-term plasticity in cochlea

3.1.1. Facilitating efferent inhibition in hair cells—Short-term plasticity refers to changes in synaptic efficacy that are induced by repetitive stimulation. Such effects can last from a few milliseconds to a few tens of seconds. Different forms of short-term plasticity elicited by various pattern of stimulation, and differing by their duration have been described: synaptic facilitation, depression, post-tetanic potentiation and augmentation (reviewed by Capogna (1998), Catterall and Few (2008)). Synaptic facilitation is the only form of short-term plasticity described so far in cochlear efferents. Single afferent recordings in the cochlea of the cat and the lateral line of fish showed that the strength of inhibition depended on the rate of electrical stimulation of efferent axons (Wiederhold, 1970; Wiederhold and Kiang, 1970; Flock and Russell, 1973). Similar results were found in the guinea pig where high rates of efferent stimulation were required to suppress the CAP and the IHC receptor potential (Brown and Nuttall, 1984). Synaptic facilitation was observed directly by intracellular recording from hair cells in the turtle's inner ear (Art et al., 1984) and in rat IHCs (Goutman et al., 2005). These intracellular hair cell recordings showed that the probability of transmitter release was low for efferent action potentials occurring at 1 Hz (probability of release from 0.08 to 0.35) but increased with higher frequency efferent activity. Presynaptic facilitation was demonstrated directly with paired-pulse intervals of 10–100 ms. In this protocol the presynaptic accumulation of calcium between pulses is thought to increase release probability, and facilitation is more prominent in synapses with an initially low probability of release (Katz and Miledi, 1968). Paired-pulse stimulation of efferent synapses on rat IHCs increased the probability of synaptic release by 150–250% (for intervals of 10–100 ms) (Fig. 3). Thus, MOC to IHC facilitation includes an increase in the probability of transmitter release. It remains to be determined if this facilitation is due to presynaptic calcium accumulation. In addition, the possibility remains that postsynaptic changes contribute to enhanced summation during repetitive efferent activity. For example, the decay of synaptic current following an efferent train is many times slower than that following a single shock (Goutman et al., 2005). This prolonged time course could result from ‘delayed release’ from the efferent terminal, but also could arise by CICR from the synaptic cistern and other hair cell calcium stores.

Intracellular recordings of the efferent synaptic effect on OHCs are more difficult to obtain. These form later than the transient MOC to IHC synapses, when the cochlea is further ossified, and hair cells become more fragile. Electrical stimulation to produce evoked release has proven particularly difficult, perhaps owing to damage to the efferent axons crossing the tunnel of Corti. Those challenges notwithstanding, preliminary reports and work in progress do show that MOC transmission to OHCs exhibits presynaptic facilitation (Ballesterio et al., ARO #95, 2010). When compared to equivalent measures of the MOC to IHC synapse (Goutman et al., 2005) the resting probability of release from MOC to OHC (~ 0.3) was significantly lower than that of the MOC to IHC synapse (~ 1.0). The probability of release from the MOC to OHC synapse more than doubled for paired-pulse intervals of 10 ms (Ballesterio et al., ARO #95, 2010), a larger change than that reported for the MOC to IHC synapse (~ 1.8 -fold). These effects also can be seen in the average IPSCs during a train of MOC shocks (Fig. 4). The initial response of the OHC is smaller, but increases more

rapidly than that of the IHC, consistent with the MOC to OHC synapse having a lower resting probability of release, and stronger facilitation.

3.1.2. Voltage-gated ion channels that support and regulate MOC transmitter release—Although the molecular mechanisms of short-term plasticity are still a topic of debate, they are all calcium-dependent (Katz and Miledi, 1968; Zucker and Regehr, 2002), and in some instances involve modification of presynaptic calcium channels. Thus it is of interest to identify the voltage-gated calcium channels that mediate neurotransmitter release from the MOC efferent terminals. Specific antagonists of P/Q, N and L-type voltage-gated calcium channels (VGCCs) were used to alter cholinergic postsynaptic currents electrically evoked in mouse IHCs (Zorrilla de San Martín et al., 2010). These experiments showed that N- and P/Q type voltage-gated calcium channels (VGCCs) support transmitter release at the MOC-IHC synapse; while L-type calcium channels activate calcium-dependent BK channels to suppress ACh release (Fig. 5). Although the specific identity of the L-type VGCCs was not determined, $\text{Ca}_v 1.2$ channels have been localized in the mouse MOC-OHC synapse and could be involved (Waka et al., 2003). The role of N- and P/Q VGCCs in transmitter release and in synaptic plasticity has been extensively described in many mammalian synapses of the peripheral (N-type) and central nervous system (P/Q type) (see for recent review (Catterall and Few, 2008)).

The relative contribution of N and P/Q type channels to efferent synapses on OHCs remains to be determined, and whether this contribution varies during development as found for other synapses (Rosato Siri and Uchitel, 1999; Iwasaki et al., 2000; Rosato-Siri et al., 2002). Also, one could imagine that the nature or contribution of VGCCs differs in MOC terminals on OHCs and IHCs, perhaps contributing to the differential efficacy of transmitter release at these two contacts. Ultimately, a comprehensive picture of MOC plasticity also will require investigation of the regulation of these presynaptic calcium channels by calcium/calmodulin as well as by neurotransmitter-mediated activation of G proteins or other regulatory proteins (see for review Catterall and Few, 2008). In particular, G protein-coupled receptors for ACh, glutamate, GABA and neuropeptides are known to inhibit VGCCs and reduce transmitter release (Hille, 1992, 1994; Ikeda, 1996). The presence of G proteins in the cochlea (Kurc et al., 1998) and G protein-coupled receptors in MOC terminals (Bartolami et al., 1993; Maison et al., 2010) suggest their role in synaptic modulation (see Section 3.1.3). Preliminary studies have shown that transmitter release at the MOC to IHC synapse can be reduced by activation of metabotropic GABA_B receptors (Wedemeyer et al., ARO #96, 2010).

ACh release from MOC efferents on IHCs is reduced by calcium flux through L-type VGCCs coupled to BK channels (Zorrilla de San Martín et al., 2010). BK channels have often been described as key regulators of neurotransmitter release in the brain (Knaus et al., 1996; Misonou et al., 2006), the neuromuscular junction (Robitaille et al., 1993) and in sensory systems (Roberts et al., 1990; Issa and Hudspeth, 1994; Grimes et al., 2009; for review see (Wang, 2008) where they have been associated with all types of VGCCs. Although their roles in MOC efferents are not fully resolved, by limiting depolarization and calcium influx they could help maintain the normally low resting probability of release. It is interesting as well to speculate that BK suppression of efferent transmitter release may be involved in the ultimate developmental loss of these synapses from inner hair cells. The timing of IHC action potentials by efferent inhibition is another link in the complex patterns of activity that are thought to be critical for the establishment and refinement of afferent connections onto IHCs and throughout the auditory system (Tritsch and Bergles, 2010; Tritsch et al., 2010).

3.1.3. Functional role of GABAergic and muscarinic receptors?—While cholinergic efferent transmission is relatively well characterized, a role for GABA as an

MOC efferent neurotransmitter is less certain. Likewise, the role of muscarinic receptors localized on efferent terminals is still unknown. As mentioned earlier, activation of cholinergic efferent fibers terminating on OHCs decreases cochlear amplification thereby elevating acoustic thresholds. This effect disappears in mice lacking $\alpha 9$ or $\alpha 10$ nicotinic receptors (Vetter et al., 2007, 1999) supporting the dominant role of ACh in efferent inhibition. Mice lacking α or β subunits of the GABA_A receptor did not show a clear cut function for these receptors (Maison et al., 2006). However, elevated acoustic thresholds were observed in $\alpha 5$, $\beta 2$ and $\beta 3$ null mice, indicative of hair cell dysfunction. Also, there was a progressive loss of efferent innervation in $\alpha 5$ and $\beta 2$ knockouts, taken to indicate a role for GABA in the maintenance of MOC innervation.

Further studies are needed to understand this long-term effect of GABA. There are suggestions that GABAergic interactions are likely to be more complex than a straightforward inhibition of OHCs. Recently, type II afferents contacting OHCs have been shown to express GABA_{B1} receptors, suggesting that their activity could be altered if MOC efferents were to release GABA (Maison et al., 2009). In a similar approach, the role of muscarinic receptors in MOC efferents was studied by looking at the physiology and morphology of mice with targeted deletions of (m1–m5) AChRs expressed in the cochlea (Maison et al., 2010). Mice lacking the Gq-coupled M1, M3, M5 muscarinic receptors exhibited normal ABR and DPOAE thresholds, as well as normal vulnerability to acoustic injury. In contrast, knockout of the Gi-coupled M2/M4 receptors markedly decreased the threshold shift in DPOAEs normally caused by loud sound exposure. This increased resistance to acoustic trauma may result from loss of presynaptic M2 receptors normally acting to suppress MOC release of ACh. The localization of M2 receptors in MOC efferent terminals by immunolabeling supports this hypothesis but further experiments are required to identify a molecular mechanism for modulation of neurotransmission by muscarinic receptors (Fig. 6).

3.2. Properties of efferent ionic channels/receptors and intrinsic plasticity

Although modulation of presynaptic transmitter release is the major source of short-term plasticity, regulation of postsynaptic ligand-gated receptors and ionic channels also can shape neurotransmitter-induced responses. Hence, as mentioned in Section 2.2, gating of postsynaptic $\alpha 9\alpha 10$ -containing nAChRs is regulated by extracellular divalent cations (Weisstaub et al., 2002; Gómez-Casati et al., 2005) and can be blocked by endogenous opioids peptides that could be co-released with ACh upon stimulation of cochlear and vestibular efferent fibers (Lioudyno et al., 2002). The efferent synaptic cistern could provide another source of postsynaptic modulation. Whether this structure serves as source or sink, the interaction of calcium release channels, ATP-dependent pumps and store-operated calcium channels in the plasma membrane could significantly shape the cytoplasmic calcium signal that is the proximal cause of hair cell hyperpolarization. A large body of data from different tissues suggests that calcium release channels are modulated by numerous physiological agents (calcium, magnesium and ATP), cellular processes (phosphorylation, oxidation.) and closely associated proteins (calmodulin, FK-506 binding proteins...) (for review see Fill and Copello, 2002; Verkhratsky, 2005).

The SK channels that mediate hair cell inhibition are activated by rise in intracellular calcium ($K_D \sim 0.5 \mu M$) and lack voltage-dependence (Park, 1994; Kohler et al., 1996; Soh and Park, 2002). Calcium sensitivity is conferred by calmodulin (CaM) constitutively bounded to the C terminus (Xia et al., 1998). Calcium binding to CaM changes SK conformation to open the channel and allow K^+ efflux. SK channels also are known to support the after hyperpolarisation (AHP) that follows an action potential (Stocker et al., 1999) but also control dendritic calcium levels (Cai et al., 2004) as well as synaptic

transmission and plasticity (for review see Bond et al., 2005; Faber and Sah, 2007; Faber et al., 2008; Faber, 2009).

Recent studies describe a macromolecular complex in which the calcium source, SK2 channels and modulatory proteins are assembled to activate and shape the kinetics of the ensuing hyperpolarization. Hence, in addition to bound calmodulin, SK2 co-assembles with the catalytic and regulatory subunits of casein kinase 2 (CK2) and protein phosphatase 2A (PP2A) that directly and dynamically regulate the channel (Bildl et al., 2004; Allen et al., 2007). Within this complex, CK2 (activated by the N terminal of SK2) phosphorylates calmodulin at threonine 80, reducing 5-fold the calcium sensitivity and accelerating channel deactivation. Dephosphorylation of calmodulin by PP2A opposes this action when CK2 is inactivated (Bildl et al., 2004; Allen et al., 2007). This process is itself calcium-dependent, whereby the higher the calcium concentration, the less CK2 is activated and the more sensitive the SK2 channel becomes to calcium. Thus the calcium sensitivity of the channel may be tuned in response to neuronal activity (efferent strength) and alter the effect of SK2 channels on the membrane potential.

Interestingly, CK2 co-localizes with SK2 at MOC synapses on OHCs in the mouse cochlea. Removal of ATP that normally maintains kinase activity causes a progressive slowing of the decay of efferent IPSCs in OHCs, suggesting that CK2 controls the gating properties of SK2 via phosphorylation of CaM (Bildl et al., 2004). Experiments using pharmacological tools that specifically target CK2 and PP2A could help determine whether this mechanism contributes to the cholinergic inhibition of hair cells. Neurotransmitter modulation of SK gating has been described in sympathetic superior cervical ganglion (SCG) neurons where norepinephrine induces a 3-fold reduction in steady-state calcium sensitivity, increasing neuronal excitability (Maingret et al., 2008). Notably, endoplasmic reticulum (ER) calcium release through RyRs or IP3Rs has been shown to facilitate functional coupling with mitochondria and to stimulate the calcium-sensitive step of ATP production (Putney and Thomas, 2006; Gunter et al., 2004; Colegrove et al., 2000). Such coupling at the MOC efferent synapse could prove important for the function of CK2/PP2A, with ATP supporting the CK2-mediated modulation of SK2 channels.

Regulation of SK channel expression and function acquires additional interest from evidence for the participation of BK (large conductance, voltage-gated, calcium-sensitive) potassium channels in cholinergic inhibition of OHCs in basal turns of the rat cochlea. BK channels were proposed to play a role in basal OHC function on the basis of expression patterns (Engel et al., 2006) and hair cell loss in knockout mice (Rüttiger et al., 2004). Although both BK and SK channel immunolabel is found in basal OHCs, responses to exogenous ACh were preferentially sensitive to iberiotoxin, a BK channel blocker, and not to apamin, selective for SK channels (Wersinger et al., 2010). A major fraction of the voltage-gated potassium current in basal OHCs also was blocked by iberiotoxin. In contrast, neither voltage-gated, nor ACh-activated currents in apical OHCs were sensitive to iberiotoxin. ACh-activated currents were completely blocked by apamin.

3.3. Modulation of postsynaptic calcium signaling

The diffusible second messenger nitric oxide (NO) is recognized as an important synaptic modulator in a variety of animals from amphibians to mammals (Mustafa et al., 2009). Both NO and its synthetic enzyme, nitric oxide synthase (NOS) are present in cochlear and vestibular hair cells and in afferent and efferent terminals (Fessenden et al., 1994; Fessenden and Schacht, 1998; Hess et al., 1998; Lysakowski and Singer, 2000; Shi et al., 2001). NO has been shown to have opposing effects on the membrane potential of type I hair cells in rat crista ampullaris. NO inhibited a large conductance outward rectifier K⁺ current (Chen and Eatock, 2000) but also reduced the magnitude of voltage-gated calcium current (Almanza et

al., 2007). The modulatory effect of NO on voltage-gated L-type calcium channels and calcium-activated BK channels has been confirmed in recent studies on frog saccular hair cells (Lv et al., 2010). ACh stimulated a rapid and sustained release of NO that required extracellular calcium, which could enter through $\alpha 9\alpha 10$ receptors.

Synaptic cisterns adjacent to the efferent terminal are suggested to be involved in a CICR mechanism that supports long lasting efferent inhibition. As mentioned earlier, this could happen through the expression of calcium release channels such as RyRs. Another striking feature of the synaptic cistern is the physical relationship with mitochondria that is often seen (Fig. 2). Whether this association is functionally relevant is currently unknown but because mitochondria can transport calcium, this coupling could have an impact on local calcium signaling at the hair cell efferent synapse, for instance in response to prolonged efferent stimulation, producing further delayed inhibition.

There is mounting evidence that mitochondria interact physically as well as functionally with endoplasmic reticulum in various cell types (Rizzuto et al., 1998). Calcium signals through RyRs can be transmitted to mitochondria in neurons and muscle cells (Szalai et al., 2000; Pacher et al., 2000; Nassar and Simpson, 2000) and mitochondria are capable of refilling the endoplasmic reticulum with calcium to prevent its depletion (Arnaudeau et al., 2001; Malli et al., 2005). Overall, the coupling between the two organelles strongly shapes calcium signals and depending on the cellular context, can either potentiate or inhibit calcium oscillations. Although these properties have been tested mostly in cell lines, similar results have been shown recently in retinal amacrine cells where mitochondrial calcium transport affects the duration of glutamate-induced calcium elevations (Sen et al., 2007). Finally, some studies have suggested that mitochondria may contain nitric oxide synthase (NOS) and produce NO in significant quantities in order to regulate metabolism and other physiological functions (Brown and Borutaite, 1999; Brown, 2000). In addition to its postulated role in efferent inhibition (Lv et al., 2010), NO produced by mitochondria also may lead to OHC pathology, contributing to noise induced hearing loss (Shi et al., 2002, 2007).

It is of interest to ask whether the ultrastructure of the SC offers further insight into possible functional roles. Deiters' cells innervated by putative cholinergic MOC efferent terminals in various species (Sobkowicz and Emmerling, 1989; Liberman et al., 1990; Nadol and Burgess, 1994) also have well-defined SR co-extensive with the efferent contact (Bruce et al., 2000). Interestingly, SK2 and $\alpha 9\alpha 10$ expression is restricted to hair cells (Elgoyhen et al., 1994, 2001; Oliver et al., 2000) which would suggest that the presence of SC is not correlated with the expression at the plasma membrane of specific molecular components. Additionally, cisterns have been located postsynaptically on type II afferent boutons (Thiers et al., 2008) and could play a role in putative reciprocal synapses between OHCs and type II afferents (Nadol, 1981, 1984). It is also interesting to ask whether cistern structure alters with synaptic function, after sustained efferent activation for example. Alternatively, one could explore genetic models with loss ($\alpha 9$ knockout) or enhancement ($\alpha 9L9^T$ knockin) of efferent function to look for correlated changes in cisternal structure.

4. Effect of altering MOC efferent function or innervation

Efferent function *in vivo* has been studied by surgical ablation, as well as through genetic elimination of essential components. Severing the efferent supply of newborn cats altered afferent response properties (Walsh et al., 1998) but not cochlear afferent innervation, (Liberman et al., 2000) although previous efforts did show elevated afferent innervation to OHCs (Pujol and Carlier, 1982). While potentially revealing, surgical ablation presents substantial experimental challenges. Thus, the alternative has been to study efferent function

in vivo using genetically-altered mice. Olivocochlear innervation and function has been studied in 'knockout' mice lacking $\alpha 9$ or $\alpha 10$ subunits (Vetter et al., 1999,2007) or SK2 channels (Kong et al., 2008; Murthy et al., 2009). These studies have largely reinforced our understanding of the mechanism leading to the cholinergic inhibition of hair cells and the subsequent effect on cochlear mechanics. Intracellular recordings from hair cells showed no detectable ACh-gated currents in the absence of $\alpha 9$ and little or no current without $\alpha 10$ subunits. Stimulation of olivocochlear fibers failed to suppress DPOAEs in both knockouts (See Fig.1c), suggesting that residual homomeric nAChRs alone cannot compensate for the absent subunit. Additionally, after silencing $\alpha 9$ or $\alpha 10$ subunits, efferent terminals still contact OHCs but appear somewhat larger in size and fewer in number suggesting some role in the maintenance of normal connections between efferent fibers and hair cells (Vetter et al., 2007). Absence of the $\alpha 10$ subunit does not induce any particular changes in electrical properties of IHCs suggesting that the $\alpha 10$ nAChR subunit is not essential for development of the intrinsic electrical properties of hair cells (Gómez-Casati et al., 2009). Interestingly, loss of the $\alpha 9$ subunit results in increased mRNA expression for components of GABA transmission, suggesting a possible association between cholinergic and GABAergic MOC efferent transmission (Turcan et al., 2010).

Similarly to $\alpha 9$ and $\alpha 10$ KO, genetic deletion of SK2 channels alters olivocochlear physiology and morphology. In contrast to the expectation that ACh would produce an excitatory effect on hair cells in the absence of SK2 channels, SK2-knockout IHCs are entirely unresponsive to ACh (Kong et al., 2008). Deletion of SK2 also severely disrupts olivocochlear innervation, suggesting a role in the establishment and/or maintenance of olivocochlear synapses (Kong et al., 2008; Murthy et al., 2009). In addition, absence of SK2 expression in IHCs during development resulted in altered excitability and impaired exocytosis (Johnson et al., 2007). It would be interesting to look at other aspects of cochlear innervation, such as synaptogenesis of type I and type II afferents in these efferent-deficient mice.

5. Role of the olivocochlear efferents in hearing

While our knowledge of the cellular mechanisms of MOC efferent physiology has grown considerably in the past two decades, the integration of this knowledge into a broader understanding of audition is a still more challenging task. For example, MOC efferents have been suggested to shift the dynamic range of hearing and to increase the ability to discriminate sounds in a noisy background (anti-masking effect) (for review see Christopher Kirk and Smith, 2003; Guinan, 2006). However, mice lacking $\alpha 9$ nAChR have no MOC inhibition of hair cells, but behaved normally in signal-in-noise sound-localization tests (May et al., 2002).

5.1. Efferent protection against acoustic trauma

Experimental studies suggest that efferent feedback protects against acoustic injury (Rajan and Johnstone, 1988; Rajan et al., 1991; Kujawa and Liberman, 1997; see review from (Rajan, 2000)). Animals with weak MOC reflexes were the most vulnerable to noise injury, whereas those with the strongest reflexes suffered the least damage (Maison and Liberman, 2000). This protection might depend on the ACh-mediated slow effect (Sridhar et al., 1995) as it only occurs in regions tuned for high frequencies (where the slow effect predominates) and disappears with prolonged efferent stimulation known to extinguish the slow effect (Reiter and Liberman, 1995). In keeping with the hypothesis of efferent protection, over-expression of $\alpha 9$ nAChRs in OHCs enhances the electrically-evoked olivocochlear effect, and protected these animals from temporary and permanent acoustic injury (Maison et al., 2002). Similarly, mice with a point mutation in $\alpha 9$ nAChRs that increases the gating and open time

of the nicotinic receptor, thereby increasing efferent synaptic strength, were more resistant to permanent acoustic injury (Taranda et al., 2009).

5.2. MOC firing patterns and efferent-mediated protection against acoustic injury

Efferent studies in mammals generally use high frequency electrical stimulation (100–400 Hz) to achieve maximal inhibition. How does this compare to efferent activity elicited by acoustic stimulation? Spontaneous discharge rates of MOC neurons in the absence of sound stimulation are known to be very low (Lieberman, 1988) but can increase up to 80 Hz in response to monaural stimulation with tones (~90 dB SPL in a quiet environment) in cat and guinea pig cochlea (Robertson and Gummer, 1985; Liberman and Brown, 1986; Liberman, 1988). In ‘efferent protection’ experiments, the magnitude of the protective effect is directly proportional to the intensity of the acoustic trauma, as well as its duration (Rajan and Johnstone, 1988; Rajan, 1995), implying a correlation with the acoustically-driven activity of efferent neurons.

These MOC discharge rates, although sufficient to facilitate synaptic transmission (Fig. 4), are still well below those shown to increase afferent thresholds (and to produce a protective effect) (Gifford and Guinan, 1987). However, binaural acoustic stimulation and/or the addition of noise to the opposite ear can increase MOC discharge rate still further (up to 140 Hz) (Lieberman, 1988). This binaural effect depends on intensity (increasing at high sound level, >105 dB), the nature of the sound (noise > tones) and is sensitive to pre-conditioning (Brown et al., 1998a,b). Importantly, binaural acoustic stimulation exercises protective effects at the cochlea that mimic the effects of electrical stimulation of the efferent pathway (Rajan and Johnstone, 1988).

The low resting probability of release, and marked efferent facilitation observed in all hair cells to date, including at the MOC to OHC synapse, implies that these are essential properties of feedback to the auditory periphery. If so, facilitation of efferent inhibition reflects not only the subtleties of transmitter release in a controlled experiment, but could be a necessary physiological basis for MOC efferents to achieve their biological role.

6. Conclusion

Over the past two decades, considerable progress has been made in determining the cellular and molecular mechanisms of inhibitory feedback onto sensory hair cells. Although ACh is known to support efferent-mediated hair cell inhibition, there are several other neurotransmitters (GABA, CGRP, opioids) colocalized with ACh in the MOC efferent terminals, suggesting modulation of synaptic transmission and the possibility of different forms of synaptic plasticity. The molecular components as well as the mechanisms of such modulation remain to be identified in future studies. In addition to neurotransmitters, G proteins and intracellular second messengers acting on presynaptic calcium channels known to support efferent neurotransmission are likely to be involved. Their identification will provide better understanding of short-term plasticity at MOC synapses, and how olivocochlear efferents modulate hearing.

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Abbreviations

MOC(s)	medial olivocochlear (efferents)
LOC(s)	lateral olivocochlear (efferents)
OHC	outer hair cell
IHC	inner hair cell
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
CAP	compound action potential
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
nAChR	nicotinic acetylcholine receptor
SK2	small conductance calcium-activated potassium channel
BK	large conductance, voltage-gated, calcium-sensitive potassium channel
GABA	gamma amino butyric acid
CGRP	calcitonin gene-related peptide
VAT	vesicular acetylcholine transporter
GAD	glutamate acid decarboxylase
VGCC	voltage-gated calcium channel
CaM	calmodulin
RyR	ryanodine receptor

Fig. 1. MOC efferent pathway and function. Schematics showing the peripheral projection of MOC fibers (a) and the efferent synapse onto OHCs in the mature organ of Corti (b). MOC efferent neurons are located in the superior olivary complex and project via myelinated fibers to make synaptic contacts directly onto the base of OHCs. Before the onset of hearing, IHCs are innervated as well (a, dashed red line). Although GABA, CGRP and opioid peptides are also present in MOC terminals, ACh is considered to be the primary transmitter of the efferents. ACh released by efferents binds postsynaptically to the highly calcium permeable $\alpha 9 \alpha 10$ nAChRs. This leads to the subsequent activation of calcium-dependent SK2 K^{+} channels and K^{+} efflux, hyperpolarizing the hair cell. (c) This coupling of nAChRs and calcium-gated channels is solely responsible for hair cell inhibition since both $\alpha 9$ and $\alpha 10$ knockout mice fail to show suppression of distortion product otoacoustic emissions (DPOAEs) during efferent fiber stimulation. a and b are adapted from Fig. 1., Taranda et al., 2009 and Fig. 1c is reproduced with permission from Fig. 4., Vetter et al., 2007.

Fig. 2. MOC efferent synaptic structure. (a) Transmission electron micrographs of an OHC innervated by two efferent terminals in the middle turn of a P21 mouse cochlea. Note the presence of the synaptic cistern (arrow) closely apposed to the postsynaptic membrane of the hair cell and of several mitochondria (asterisk) near the cistern. (b) High magnification of a P21 mouse efferent synapse. One can clearly see the small space (~ 14 nm) between the OHC plasma membrane and the synaptic cistern. Similarly, mitochondria are often found in close proximity to the cistern suggesting that they may have a role in maintenance or function of the efferent synapse. Image courtesy of Dr M. Lehar, the Johns Hopkins University School of Medicine.

Fig. 3.

Effect of repetitive electrical stimulation on efferent synaptic transmission and IHC excitability. (a) Average IPSCs evoked with a paired-pulse protocol (50 pulses at a rate of 0.25 Hz, 50 μ A in size and 50 μ s long; 10–25 ms intervals) showing facilitation at the IHC-efferent synapse. Facilitation of transmitter release is observed with short inter-pulse intervals (ranging from 10 to 100 ms) but does not occur with longer intervals (500 ms). Facilitation results from an increase in the probability of neurotransmitter release leading to a higher quantum content. For a 25 ms paired pulse interval (a, second panel), quantum content increases by 150–550% (not shown here) and the mean amplitude of the second evoked IPSC increased up to 200–300% compared to the first IPSC. (b) Stimulating efferent fibers at 2 Hz had no effect on calcium action potentials elicited in IHCs by injection of a +50 pA current. In contrast, repetitive efferent stimulation (at frequency $\geq$ 5 Hz) and the attendant facilitation and summation can effectively prevent calcium action potentials in IHCs. a and b are reproduced with permission from Figs. 3 and 5 respectively, Goutman et al., 2005.

Fig. 4.

Electrical stimulation of efferent synapses on inner and outer hair cells of the rat cochlea. (a) A train of 10 shocks at 40 Hz produced averaged ($n = 10$) postsynaptic currents that facilitated and summed at -90 mV in a rat inner hair cell (postnatal day 7). (b) The same shock train produced summed and facilitated (averaged, $n = 10$) IPSCs at -90 mV in a rat outer hair cell (postnatal day 18). Note that in comparison to the IHC, average synaptic currents were initially smaller, then grew more rapidly during the train in the OHC. Unpublished results from J.H.Kong & P.A. Fuchs.

Fig. 5. Identification of VGCCs expressed in IHCs-efferent terminal and modulation of synaptic transmission. (a) Representative traces of electrically-evoked IPSCs before (control, left panels) and after (right panels) bath application of 200 nM ω -agatoxin IVA (ω -Aga) or 300 nM ω -conotoxin (ω -CgTx), which block P/Q and N-type calcium channels, respectively. This suggests a role for these channels in ACh release. In these experiments, quantum content decreased by ~55% and ~45% respectively (b). Bath application of either IBTX (100 nM) which specifically blocks BK channels, or nifedipine (3 μ M) which specifically blocks L-type VGCCs increased quantum content ($243.8 \pm 45\%$ and $195.2 \pm 22.6\%$, respectively) in response to electrical stimulation of MOC efferents. Therefore, in marked contrast to the role of N- and P/Q type VGCCs, a functional coupling between calcium-activated BK channels and L-type VGCCs negatively modulates the release of ACh at the IHC-efferent synapse. Fig. 5a and b are reproduced with permission from Figs. 2 and 5. respectively, Zorrilla de San Martín et al., 2010.

Fig. 6.

Schematic representation of MOC efferent synaptic transmission and modulation. The main transmitter of the MOC efferent is ACh. At the efferent terminal, release of transmitter requires activation of P/Q- and N-type VGCCs, believed to be localized near the release site. ACh is known to activate the postsynaptic calcium-permeable $\alpha 9\alpha 10$ nAChRs leading to the activation of SK2 K^+ channels (in most hair cells) and the inhibition of hair cells. In addition, L-type VGCCs coupled to BK K^+ channels expressed in the presynaptic terminal negatively modulate efferent transmission. Whether this mechanism is conserved between the transient developmental efferent-IHC synapse and the post-hearing efferent-OHC synapse remains unknown. Neurotransmitters colocalized with ACh in efferent terminals onto OHCs (GABA, CGRP and other peptides) may act as neuromodulators of synaptic transmission by activating presynaptic receptors. For instance M2 muscarinic receptors may be activated presynaptically upon release of ACh (see Maison et al., 2010). Likewise, the postsynaptic effect of GABA is controversial but could activate receptors expressed presynaptically or localized in the type II afferents (i.e. such as G protein-coupled GABA_B receptors) (see Maison et al., 2009). Fig. 6 is adapted with permission from Fig. 7. Zorrilla de San Martín et al., 2010.